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(54) Title: DIAGNOSIS AND TREATMENT OF AUTOIMMUNE DISEASES

(57) Abstract

A treatment for testing a mammal for predisposition to develop an autoimmune disease, comprising measuring HLA class I expression on cells of said mammal, a decreased level of expression indicating said predisposition.

- 1 -

TITLE OF THE INVENTION
DIAGNOSIS AND TREATMENT OF AUTOIMMUNE DISEASES
Background of the Invention

5   This invention relates to testing and treatment of
autoimmune diseases such as type I diabetes. The
invention takes advantage of a link I have discovered
between type I diabetes and major histocompatibility
complex (MHC) class I molecules, or HLA class I

10   molecules, as they will be referred to herein.

          T-lymphocytes recognize self or foreign proteins
in the binding groove of HLA, resulting in HLA-restricted
immune responses. Peptides bound by HLA class I are
recognized by CD8+ suppressor or cytotoxic T-cells,

15   whereas peptides bound to HLA class II are recognized by
CD4+ helper T-cells. Peptides in the extracellular
compartment are taken up by antigen presenting cells by
endocytosis and subsequently are presented as peptides in
association with HLA Class II complexes. In contrast,

20   endogenously synthesized antigens, presumably as "self
peptides", are transported into the endoplasmic reticulum
where they preferentially bind to HLA class I. HLA class
I expression is normally universally present on all
cells. Recently, a series of experiments have identified

25   transmembrane transporter genes involved in the process
of transportation of cytosolic proteins into the
endoplasmic reticulum, and mapping studies have localized
their chromosomal location within the HLA class II
region. These peptide supply factor genes (also known as

30   ATP-dependent transporter protein-encoding genes), in
particular RING 4, HAM1, Mtp1, HAM2, Mtp2, and Y3 are
members of the multidrug resistant family of transporters
and are highly conserved between species. Their
identification has been feasible by a series of induced

35   mutant cell lines which lack surface HLA class I (i.e.,

- 2 -

do not "present" HLA class I) by virue of deletions in an endogenous peptide transporter gene.

Type I diabetes is an autoimmune disease characterized by T-cell mediated destruction of the beta
5   cells in the islets' of Langerhans, accompanied by an immune response to a diversity of self peptides. Many proposals have been put forth on the mechanism of self reactivity and the previously identified strong genetic associations of this disease with HLA class II genes.
10   Humans at risk for type I diabetes can be identified years prior to hyperglycemia by the abnormal occurrence of autoantibodies to insulin, islet cells, glutamic acid decarboxylase, as well as many other autologous proteins. The autoantibody patterns predict
15   eventual disease progression and/or risk. A recent analysis of "prediabetics" as well as discordant diabetic type I identical twins revealed a T-cell developmental defect controlled by abnormal autologous presentation of self antigens which was predictive of disease
20   progression.

Summary of the Invention

I have discovered that type I diabetes and other autoimmune diseases in man and mouse are accompanied by faulty expression of HLA class I molecules associated
25   with impaired antigen presentation. The T-cells of the diabetic respond to self antigens as if they were foreign antigens, mediating a defective development of self tolerance. I propose that this defect underlies beta cell autoimmunity, much as abolition of HLA class I
30   expression by beta microglobulin gene deletion in the mouse results in hyperglycemia due to a lymphocyte-mediated insulitis. My evidence suggests that the faulty low expression in HLA class I in the human type I diabetic is due to mutant genes in one or more of the
35   proteins involved in presentation of proteins or peptide

- 3 -

fragments thereof on the cell surface by complexation
with class I molecules. This failure of patients with
autoimmune diseases to properly present tolerance-
inducing self antigens can be due to, for example,
5   mutations in a class I gene, in one or more of the
peptide transporter genes, or in one or more of the genes
encoding the proteosomes responsible for cutting up
("processing") self-proteins for transport to the cell
surface for complexation and presentation with HLA class
10 I.

The discovery provides the basis for new tests for
autoimmune diseases based on faulty HLA class I
presentation, and in addition provides new methods for
inducing tolerance to any protein antigen, all based on
15 presenting the antigen to the patient bound in HLA class
I.

Accordingly, the invention provides, in a first
aspect, a method for testing a mammal, e.g., a human
patient, for predisposition to develop an autoimmune
20 disease, by measuring HLA class I expression on cells,
e.g., B lymphocytes, of the mammal, a decreased level of
expression indicating such predisposition. The
autoimmune disease is preferably type I diabetes, but can
also be systemic lupus erythmatosis (SLE), rheumatoid
25 arthritis, Graves disease, hypoparathyroidism,
hypothyroidism, multiple sclerosis, Addison's disease,
Celiac disease, Sicca syndrome, Addison's, Myasthenia
gravis, Idiopathic mantraneous nephropathy, Optic
neuritis, Goodpasteur's Syndrome, Pemphigus, Hashimoto's
30 thyroiditis, pernicious anemia, or ankylosing
spondylitis.

In a related aspect, the invention provides a
method for testing a mammal for predisposition to develop
an autoimmune disease by first obtaining a biological
35 sample from the mammal, and then determining, for that

- 4 -

sample, whether there is a defect in or deletion of a
gene encoding a component of class I or a protein which
is involved in the processing or transport of endogenous
proteins into the endoplasmic reticulum for HLA class I
5 presentation, or a protein involved in one of the other
steps necessary for class I presentation. In preferred
embodiments of this method, the mammal is a human fetus,
and the protein is an ATP-dependent transporter protein
(i.e., a peptide supply factor protein) or a proteosome
10 or component thereof. The determination of a defective
or deleted gene can be carried out in any conventional
manner, such as by Western blot analysis, mRNA Northern
blot analysis, cell surface protein phenotyping, or
restriction fragment length polymorphism (RFLP) analysis,
15 or polymerase chain-reaction (PCR).
             My discovery also provides the basis for a method
of treating a mammal such as a human patient to inhibit
development of an autoimmune disease; preferably such
treatment if carried out at an early stage, when
20 tolerance is most easily induced. The method involves
increasing the amount of HLA class I complexed with self-
antigen presented on circulating cells in the mammal.
One method of achieving this is to increase the amount of
HLA class I presented on circulating cells of the mammal.
25 This increased presentation of self-antigen complexed
with HLA class I allows the proper presentation of self-
antigens on cells of the mammal, increasing self-
tolerance and decreasing the tendency to develop the
autoimmune disease. This increase in HLA class I
30 presentation can be achieved in a variety of ways. In
one preferred embodiment, the mammal is treated with
cells which present a HLA class I bound to endogenous
proteins or fragments thereof. Those cells can be
autologous cells, e.g., the patient's lymphocytes (e.g.,
35 B cells or macrophages) which are transfected with DNA

- 5 -

encoding one or more of the proteins involved in the
processing or transport of endogenous proteins into the
endoplasmic reticulum for HLA class I presentation or
transport and processing of this complex to a successful
5   journey to the cell surface; these proteins are
preferably the ATP-dependent transporter proteins
including RING 3, RING 4, RING 11, HAM 1, HAM 2, Mtp 1,
Mtp 2, or Y 3 (the terminology of these transporter
proteins varies somewhat from species to species, and in
10   addition is not universally agreed upon as of yet;
although terminology varies between species, the evidence
thus far suggests that the homology of these proteins
between species is extremely high) or the cutting
proteins (the proteosome complex) responsible for
15   processing endogenous peptides. The proteosome complex
is a large (approximately 250,000 mw) assembly of
enzymatically active fragments which process self-
proteins, cutting them into pieces generally between
about 6 and 14 amino acids in length, so that those
20   fragments can enter the endoplasmic reticulum for
transport to the surface for complexation with class I.
The proteosome genes are linked to HLA class II, and are
described, e.g. in Kelly et al. (1991) Nature, page____.
Alternatively, the cells can be ones which have been
25   stimulated with an immunostimulant to increase class I
expression.
        Another method of increasing the amount of HLA
class I presented on circulating cells of the patient is
to administer to the patient or the patient's cells in
30   culture a substance which stimulates HLA class I
expression; such substances include the interferons,
e.g., a beta- and gamma-interferon, interleukins, and
toxoids, e.g., diphtheria toxoid, tumor necrosis factor,
BCG, and pertussus toxoid. Infection with a virus or

bacterium which stimulates HLA class I presentation is another method which can be employed.

Another method for increasing HLA class I presentation in cells of the patient is to obtain HLA
5 class I-presenting cells from another human (preferably one who has been type-matched) and administering those cells to the patient. An alternative strategy is to administer to the mammal the self-antigen or fragment thereof which forms a complex with class I on the surface
10 of a cell, to both stabilize class I and present the antigen to the immune system of the mammal. Preferably, the antigen is a peptide fragment of a protein; preferably the peptide is 6 to 14 amino acids long, the size range of peptide fragments known to complex with
15 class I. The peptide can be administered orally or intravenously, or cells from the mammal can be incubated with the peptide and then reinfused into the mammal.

The discovery of the role of class I in induction of self-tolerance can make possible the reduction of
20 tolerance in a patient to any protein, whether self or exogenous; induction of tolerance is achieved by administering to the patient either cells on which the protein to which tolerance is to be induced is presented bound to HLA class I, or antigen-class I complex itself.
25 The method can be used, e.g., where the patient is the recipient of an allograft such as a heart or kidney from another patient, or even from another species. One method which can be employed is to transfect HLA class I-presenting cells, preferably autologous cells such as the
30 patient's B cells, with DNA encoding the protein to which tolerance is to be induced, and then introducing those cells into the patient, where self-tolerance will be induced by virtue of HLA class I presentation of the protein.

- 7 -

Other features and advantages of the invention will be apparent from the following description of the preferred embodiments thereof, and from the claims.

Detailed Description

5   The drawings are first described.

Drawings

Fig. 1 is a graph showing relative HLA class I on lymphocytes from diabetic and control individuals.

Fig. 2 is a set of graphs showing that NOD mice (a
10 model for diabetes) have splenocytes markedly reduced in expression of HLA class I antigen.

Fig. 3 is a photomicrograph of an islet from a β_2 microglobulin deficient mouse showing lymphocyte infiltration.

15   Fig. 4 is a set of FACS-generated graphs showing reactivity of normal and diabetic mouse lymphocytes with antibodies to isoforms of CD45.

Fig. 5 is a Northern blot showing the lack of expression of the RING 4 ATP-dependent transporter
20 protein in diabetic lymphocytes compared to normal expression in a human control.

Fig. 6 is an RFLP Southern blot from the NOD mouse comparing DNA encoding an ATP-dependent transporter protein in lymphocytes from normal and diabetic patients.
25 The figure clearly shows the large deletion in RING 4 in the diabetic animal.

Fig. 7 is a bar graph showing decreased class I expression on cells taken from patients with various autoimmune diseases.

30   There first are described experiments demonstrating that prediabetic and diabetic mice and humans have decreased HLA class I expression.

- 8 -

"Prediabetic" and Diabetic NOD Mice and Humans have Decreased HLA Class I Expression

Using cell surface phenotyping with an HLA class I-specific monoclonal antibody (W6/32) the peripheral

5   blood lymphocytes from six high risk prediabetics whose serum contained autoantibodies to both insulin and islets; four diabetic twins; and twenty long-term diabetics were assayed for HLA class I expression. The human lymphocytes were prepared for immunofluorescence by

10   standard techniques using a ficol gradient. Flow cytometry (FACS) was carried out with a gate set which excluded remaining red blood cells and debris and which included T cells, B cells, and macrophages. HLA class I expression was also measured in ten Epstein-Barr virus-

15   transformed B cell lines taken from long-term type I diabetics, and several other groups of patients.

Referring to Fig. 1, HLA class I expression was significantly reduced in all six high risk diabetics, all four diabetic twins, and nineteen of twenty long-term

20   diabetics, and was reduced as well in all ten of the EBV-transformed B cell lines from long-term diabetics.

In marked contrast, all five low-risk prediabetics (i.e., those with insulin autoantibodies, but not islet autoantibodies); four non-diabetic discordant type I

25   twins; ten first-degree relatives; thirty-nine controlled individuals; and ten EBV-transformed cell lines from normal subjects demonstrated normal levels of HLA class I antigen expression; these levels were significantly higher than the other groups. The twin results

30   demonstrate that class I surface expression can be independent of genotype.

The NOD mouse represents a well-characterized model for type I diabetes with a similar production of autoantibodies to insulin and/or islet cells weeks prior

35   to frank hyperglycemia, as well as chronic lymphocytic

- 9 -

infiltrates surrounding the islets prior to islet
destruction. The HLA class I haplotype of the NOD mouse
(H-2 loci) is H-2K^d and H-2D^b. "Prediabetic" NOD
splenocytes were analyzed by flow cytometry. Splenocytes
5   were tested 6 and 20 weeks prior to frank hyperglycemia.
Referring to Fig. 2, in ten NOD mice, 6+/-2.3% of the
splenocytes bound to the H-2K^d monoclonal antibody clone
(31-3-45) compared to 88+/-15.8% positive splenocyte from
positive control BALB/c mice. The mean antigen density
10 for H-2K^d was also significantly reduced for the NOD
splenocytes compared to BALB/c splenocytes (p=.001). A
similar reduction in H-2D^b expression on NOD splenocytes
was also present. Using monoclonal antibody H141-31
directed to
15 H-2D^b, 52+/-15% of NOD splenocytes were positive compared
to 94+/-5.6% positive splenocytes from C57BL/6 mice (H-
2^b) (n=10). H-2^d haplotype BALB/c mice (n=10) were 38+/-
7.8% positive with this H-2D^b directed antibody,
demonstrating that the NOD splenocytes were also severely
20 reduced in the expression of this HLA class I antigen.
Therefore, in both the human as well as the mouse,
decreased HLA class I expression was observed on
splenocytes in the NOD mouse and peripheral blood
lymphocytes from diabetic humans.
25 **Decreased HLA Class I Expressions in Other**
Autoimmune Diseases
        The procedure described above, employing an HLA
class I-specific monoclonal antibody, was carried out on
peripheral blood lymphocytes from patients suffering from
30 the following autoimmune diseases: Sjogren's syndrome;
rheumatoid arthritis; type I polyendocrine failure;
multiple sclerosis; SLE; hypothyroidism; Hashimoto's
disease; and Graves' disease. In every instance there
was decreased class I expression for lymphocytes obtained
35 from patients with autoimmune disease in contrast to the

- 10 -

normal MHC class I expression in non-autoimmune type II diabetic patients.

Artificial interruption of HLA class I expression *in vivo* is sufficient for autoimmune type I diabetes

The following experiments were then carried out to determine whether defective HLA class I expression itself can lead to type I diabetes.

Mice homozygous for a β_2-micoglobulin deficiency do not express any detectable β_2 microglobulin and lack almost completely H-2K and partial H-2D major histocompatibility antigens on their cell surfaces. Ten mice over a year of age were checked for hyperglycemia, as well as body weight. All ten homozygous deficient mice greater than 1 1/2 years of age were hyperglycemic as well as having a significant decrease in body weight compared to normal littermates of the same age (Table 1).

TABLE 1

Blood Sugar and Body Weight of HLA class I Deficient Mice Due to Lack of B_2-Microglobulin

Genotype of Progeny

	- / -			+/ -	
5	BS (mg%)	Wt (gms)		BS (mg%)	Wt (gms)
	239	22		92	37
	410	18		81	42
	333	16		76	31
	375	24		65	31
10	396	27		62	35
	401	18		71	41
	339	21		80	32
	368	21		82	38
	347	26		77	37
15	397	26		79	49

Mean +/- S.D. 360+/-50   21.9+/-3.8   79+/-8.73   37.3+/-5.6

(+/+ mice were not diabetic and had normal blood sugar and body weight)

- 12 -

Histology was carried out on an islet cell of one of the mice homozygous for β_2 microglobulin deficiency, using hemotoxylin and eosin staining. Fig. 3 shows that the islet is surrounded with CD4+ lymphocytic

5 infiltrates, as is typical of diabetic mice and humans. At the time of autopsy this mouse had an elevated blood sugar of 345 mg% and the few remaining islets were obscured by lymphocyte foci.

A test of the serum from a diabetic mouse revealed

10 lymphocyte infiltration, providing further evidence for the autoimmune mechanism of this disease. Furthermore, since these homologous recombinant mice lack CD8 cells, this new model of autoimmunity suggests that islet destruction can be mediated without CD8 cells, thus

15 suggesting a central and possibly exclusive role of CD4 or natural killer cell mediated islet attack. Although it appears that the onset of elevated blood sugars occurs after a year of age, a time point later than the hyperglycemia in the NOD mouse, the data clearly

20 establishes the functional importance of HLA class I in the establishment of tolerance to self; moreover, the presence of a global defect in the presentation of self peptides on HLA class I is sufficient for the manifestations of a very focal form of clinically

25 detectable autoimmunity, type I diabetes.

Decreased HLA class I is associated with _in vitro_ cytotoxicity to self: A lesson from discordant type I diabetic twins

The following experiments were carried out to

30 determine whether the reduced HLA class I molecules expressed on diabetic cells were phenotypically normal or impaired functionally in the presentation of endogenous antigens. It has previously been noted that macrophages and B-cells from a non-diabetic identical twin, when

35 cultured with T-cells from the syngeneic diabetic twin,

- 13 -

demonstrated augmented proliferation compared to the same
incubation carried out with autologous components from
either donor (i.e., an autologous mixed lymphocyte
reaction-AMLR). In contrast, it was previously shown
5   that the non-T-cells of the diabetic twin elicited less
stimulation of the non-diabetic twin T-cells than does
the autologous non-diabetic antigen presenting cells. It
had also been shown that diabetic twin T-cell
proliferation to HLA identical non-diabetic twin
10   stimulators substantially exceeds the suppressed diabetic
AMLR and also exceeds slightly control T-cell
proliferation to self.

An explanation for these observations which is
consistent with the discovery of the role of HLA class I
15   in autoimmunity is that the somewhat hyper-responsive but
"syngeneic" mixed lymphocyte reaction observed with non-
diabetic antigen presenting cells and diabetic T-cells
really represented an attenuated alloantigen response as
in the mixed lymphocyte reaction (MLR) occurring because
20   proper presentation of previously unrecognized endogenous
peptides is provided by HLA class I on the non-diabetic
stimulators. The diabetic twin T-cells would, therefore,
recognize the non-diabetic twin peptides, now properly
presented, as foreign due to the lack of previous
25   exposure and tolerance induction.

A characteristic outcome of the MLR, not observed
in an AMLR, is the generation of cytotoxic effector
cells. To determine whether the syngenic MLR between
non-diabetic antigen presenting cells and diabetic T-
30   cells generated cytotoxic effectors, the following assay
was performed. An AMLR with interchanges in stimulator
cells was set-up between diabetic discordant twin pairs.
A representative assay is shown in Table 2. After seven
days of AMLR proliferation, the responding T-cells were
35   harvested and used as possible autoreactive T-cells

- 14 -

against chromium labelled self and twin targets in secondary cytotoxic T-lymphocyte assays. The data in Table 2 shows that diabetic twin T-cells demonstrated excessive proliferation in the AMLR to non-diabetic twin
5 stimulators lysed "syngeneic" twin targets but not self targets. As predicted, diabetic twin T-cells failed to generate autotoxicity to diabetic targets and autologous stimulated non-diabetic twin T-cells failed to lyse self. These results suggested that the previously observed
10 excessive diabetic twin T-cell proliferation from co-culture with irradiated non-diabetic twin stimulators was secondary to the presentation of previously unrecognized self peptides. Most significantly, the autologous CTL production could be blocked by concealment of target HLA
15 class I with a polyclonal HLA class I antibody, suggesting that the diabetic twin T-cell killing was directed toward the HLA class I epitope. In contrast, control CTL assays were not significantly blocked with this polyclonal antibody. These data suggest that
20 autoreactivity to self antigens was present in diabetic twin T-cells, and could be unveiled in the context of correctly presented self peptides on HLA class I positive autologous cells.

TABLE 2

PERCENT SPECIFIC Cr RELEASE FROM TARGETS

AMLR ASSAY RESPONDERS T-Cells	SIMULATOR Non-T-Cells	TARGETS	EFFECTOR TO TARGET CELL RATIO			
			1:1	5:1	10:1	40:1
A.	Diabetic Twin	Diabetic Twin	1.3+/0.4	0.9+/-0.4	4.5+/-0.3	5.4+/-0.2
	Non-diabetic Twin	Non-diabetic Twin	2.1+/-0.5	2.1+/-0.4	2.5+/-3.1	4.8+/-0.2
	Diabetic Twin	Non-diabetic Twin	16.2+/-1.9	20.1+/-1.5	40.0+/-2.0	59.0+/-6.2
	Non-diabetic Twin	Diabetic Twin	1.0+/-0.6	.9+/-0.1	2.5+/-0.1	0.2+/-0.6

TREATMENT OF TARGET CELLS WITH HLA CLASS I ANTIBODY

			1:1	5:1	10:1	40:1
B. Diabetic Twin	Non-diabetic Twin	Non-diabetic Twin	1.3+/-0.1	2.9+/-0.6	4.2+/-1.7	17.1+/-1.2

Diabetic and non-diabetic twin T-cells were stimulated for seven days at a 1:1 ratio with syageneic irradiated non-T-cells from self or identical twin as previously described [#978]. At day 7, the responding T-cells of the AMLR were harvested over Ficoll and the CTL assay performed. Target lymphocytes represented frozen lymphocytes from the donors which were thawed 24 hours prior to the assay. Targets were labelled with Na_2 CrO_4 at a concentration of 50-150μ Cl of ^{51}Cr for 1×10^6 cells at 37°C for one hour on a slow shaking platform. Target cells were washed gently two times prior to coculture with syngeneic stimulated T-cells for ten hours at 37° at the above ratios. One hundred lambdas of the culture supernatant were harvested and counted in a gamma counter at the end of the assay. The above experiment was performed with triplicates. For Part B, target cells were treated with a 1:100 dilution of sterile polyclonal mouse anti-human HLA class I serum [#978] for 30 miuntes at room temperature, wash, and added to the culture plate for the cytotoxicity assay.

- 16 -

Defective T-cell development in diabetes is secondary to altered HLA class I expression and presentation of endogenous peptides

There has previously been described in diabetic
5 humans a T-cell developmental defect with diabetic
lymphoid cells expressing a disproportional increase in
the numbers of lymphocytes expressing the low mean
antigen density of many surface markers such as CD45,
LFA-3, LFA-1, ICAM, CD2, etc., resulting in the lack of
10 or diminution in the normal second peak of brightly
fluorescent cells. In the human, the subpopulations of
lymphoid cells in the dull peak have commonly been
referred to as naive cells or suppressor inducer cells in
contrast to the brightly fluorescent cells referred to as
15 memory cells or helper-inducer cells. More recently, it
has become clear that the increased dull to bright cells
in diabetics is not only predictive of disease rate but
secondary to a block in the normal transition from naive
to memory T-cells with autologous development.

20 Murine monoclonal antibodies recognizing different
CD45 isoforms have recently defined the mouse leukocyte
common antigen. Antibody CD45R-1 (YCD45R-1) is specific
for the high molecular weight isoforms of CD45, including
exons A and B but excluding exon C. Normal BALB/c
25 peripheral blood lymphocytes from mice stained with
CD45R-1 demonstrate heterogeneity in expression,
demonstrating the expected bimodal distribution in
fluorescent intensity with dull and bright peaks (Figure
4A). In marked contrast, NOD lymphocytes from the
30 peripheral blood almost totally lack the high density
CD45R. The β_2-microglobulin disruption mice, lacking HLA
class I expression, exclusively express the low density
population of CD45R, thus suggesting the central role of
HLA class I presentation in lymphocyte maturation (Figure
35 4C). Furthermore, analysis of the NOD mouse and HLA

- 17 -

class I β_2-microglobulin deficient mouse revealed the
lack of a high density peak for ICAM and two other CD45
antibodies on all peripheral blood lymphocytes, thus
implicating the central developmental role of properly
5　presented HLA class I as the possible ligand which drives
development of peripheral lymphocytes.

**Assay for mRNA Encoding An ATP-Dependent Transporter
Protein**

　　　　Referring to Figure 5, a standard mRNA Northern
10　blot assay was carried out for detecting the expression
of one of the ATP-dependent transporter proteins, RING 4,
in a human tumor cell line, peripheral blood lymphocytes
from long-term diabetics, and a normal control. A large
amount of mRNA was detected in the human tumor cell line
15　(lane 1). There is a virtual lack of RING 4 mRNA in
peripheral blood lymphocytes from long-term diabetics
(lanes 2, 3, 5, and 6). In lymphocytes from the normal
control individual, RING 4 mRNA was present (lane 4).

RFLP Analysis

20　　　　Referring to Figure 6, RFLP analysis was carried
out on NOD mouse DNA, and the results compared to BALB/c
and C57 BL/6 control DNA, using RING 4 DNA as a probe.
DNA from spenocytes was prepared from NOD ($H-2K^d$ $I-A^d$),
BALB/c ($H-2^d$) and C57BL/6 ($H-2^b$) mice and cut with a
25　variety of enzymes followed by loading 5 ug of DNA per
lane onto an agarose gel. A southern transfer was
performed and the gene screen plus filter probed with
RING 4. DNA was run in
　　　　Lane 1, 2, 3, 4, 5, 6, 7 (Lane 8 DNA improperly ran in
30　the agarose gel and remained in the slot at the top of
the gel.) Lanes 1, 4, and 7 represent BALB/c; Lanes 2
and 5, represent C57BL/6; and lanes 3, 6 and 9 represent
NOD. The photo shows that the same probed bands could be
visualized with BstEII or BamHI in Lanes 1, 2, 3 or 4, 6,
35　7. Lane 9 demonstrates the large deletion in the ATP-

- 18 -

dependent transporter of the NOD with a significant
decrease in probe band size in Lane 9 for the NOD DNA cut
with XbaI compared to the BALB/c DNA on lane 7.

Therapy

5   As discussed above, the discoveries of the
invention make possible therapies for autoimmune
diseases, as well as therapies in which tolerance to a
particular protein antigen is desired, e.g., in
prevention of allograft rejection. Some of these
10 therapies will now be discussed in more detail.

Gene Therapy

Where a patient suffers from or has the propensity
to devlop an autoimmune disease such as type I diabetes
because of a defect in or deletion of one of the proteins
15 involved in processing or transport of self antigens for.
complexation with class I, or processing of the complex
(MHC class I; endogenous peptide and beta 2
microglobulin) successfully to the cell surface, or a
defect or deletion in a class I gene itself, one mode of
20 therapy involves transfecting cells with a missing or
defective gene and reintroducing those cells into the
patient. The functional protein in those cells will
process or transport the endogenous, cytosolic proteins
into the endoplasmic reticulum for complexation with HLA
25 class I for presentation on the cell surface, an event
which will induce self-tolerance and inhibit the
development of the autoimmune disease. This therapy
ideally is carried out prior to advanced stages of the
disease, e.g., preferably, in the case of type I
30 diabetes, in patients who are at high risk because of
anti-insulin and anti-islet antibodies, but who have not
yet undergone destruction of the islet cells.

The first step is to identify the missing or
defective gene, according to one of the methods described
35 above. Once that identification has been made, cells ·

- 19 -

which are capable of presenting antigens complexed with class I are transfected with the missing or defective gene, by standard eukaryotic transfection techniques. The transfected cells are preferably the patient's own

5   cells, and preferably are lymphoid cells such as B cells or macrophages, which are known to be antigen presenting cells. The B-cells can be transiently tranfected, so as to avoid production of a permanent cell line; in this instance, introduction of the autologous transfected B-

10 cells will need to be carried out periodically, e.g., every few months, as the B-cells die out. Alternatively, an immortalized cell line can be made from the patients B-cells, e.g., by infection with EBV. The cells could be engineered so as to make them susceptible to an

15 antibiotic, so that after they have induced tolerance in the patient, the patient can be administered the antibiotic to kill the cells and prevent them from forming neoplasms.

       Lymphocytes from other individuals can also be
20 used to induce tolerance to common self-antigens. Preferably, the lymphocytes are taken from an individual who has been HLA-matched with the patient using standard matching techniques. The individual providing the lymphocytes must be one whose HLA class I antigen

25 presentation is normal, so that the antigens not presented by the patient because of the class I presentation defect are presented on the donor cells. The donor cells can be provided as a purified fraction of serum, or in whole blood. If the lymphocyte fraction is

30 used, a monthly infusion on the order of 1 x 10^8 cells might be sufficient to induce self-tolerance and prevent development of diabetes or another autoimmune disease. Furthermore, these cells can be irradiated prior to infusion to prevent graft versus host disease or the

35 transmission of any infectious disease because dead cells

- 20 -

still present already processed antigens in the HLA class I binding cleft.

Examples of the manner in which permanent and transient B cell transfections will be carried out
5   follow.

Permanent Transfection with a Multi-Drug Resistant

Transporter

The first step is to isolate B cells or lymphocytes from a patient predisposed to or suffering
10 from an autoimmune disease because of a class I presentation defect caused by a missing or defective class I gene, proteosome gene, or ATP-dependent transporter gene. The mRNA from the patient is analyzed to identify the defective or missing gene. The patient's
15 isolated B cells are immortalized by standard procedures using EBV, producing a tumor line based on the patient's cells. Following establishment and immortalization of the cell line, the cells are made sensitive to a "suicide" antibiotic by transfection, using standard
20 techniques, with a gene conferring antibiotic sensitivity.

Transfection of the B cell line to allow expression of the missing or defective transporter protein can then be carried out as follows. Transfection
25 will be performed using a cDNA molecule encoding the missing or defective protein, which cDNA will include the 72 base pairs located upstream from the translation initiation codon, which terminates two base pairs downstream from the polyadenylation site. The cDNA will
30 be inserted into a vector such as pcDNA I/NEO (In Vitrogen) under the transcriptional control of a cytomegalovirus promoter and enhancer. Transformants will be selected for resistance to neomycin (G418). Transformants will be examined at three to four weeks
35 following transfection to identify, by flow cytometry,

- 21 -

those expressing on their surfaces HLA class I antigens;
this can be accomplished, e.g., using a monoclonal
antibody such as W6/32, which recognizes all HLA class I
of humans.

5   Other expression vectors of course can be used as
well, e.g., RSV.5 (DPT), in which cDNA transcription is
driven by the ROUS sarcomavirus 5' long-terminal repeat.
In this vector, an RSV.5 (DPT) guanine phosphoribosyl
transferase gene confers resistance to mycophenolic acid.

10   Following successful transfection and screening
for-HLA class I antigen expression, sublines-optimally
expressing such antigens will be established for
reinfusion into the patient. Following infusion and
determination that tolerance to self has been achieved,

15   the antibotic to which the cells have been made sensitive
is administered to the patient to kill the cells.

           The transfection step mentioned above can be
carried out using any standard technique. Generally, in
either of the vectors mentioned above, insertion of the

20   cDNA encoding the transporter protein can employ flanking
polylinker restriction sites such as HindIII and NotI and
XbaI. Transfection can be carried out using
electroporation apparatus (BIORAD) at 1250 volts, 3
capacitor banks, minimalized time, and maximal fall time.

25   Cells are cultured at about 5×10^5 cells per ml for at
least two days prior to electroporation. Cells at a
concentration of 5×10^6 are then suspended in 0.5 ml of
culture media (RPM1 1640/15% fetal calf serum) in
electroporation cuvettes placed in an ice water bath.

30   After electroporation, cells are maintained at room
temperature for ten minutes, resuspended in fresh culture
media, and distributed into multi-well plates. The
amount of DNA per cuvette will be between 3 and 20
micrograms.

Selection for correctly transfected cell lines
will be started on Day 5 after electroporation, using
G418 (Gibco) for pcDNA 1/NEO and mycophenolic acid
(Sigma) immediately containing Xanthine (10 micrograms
per ml) for RSV.5 (DPT). After two weeks of selection,
5 drug resistant cell populations proliferating in many
wells will be examined individually. Analysis of the
transfectant cell populations by flow cytometry will
begin five weeks after electroporation. Cells will be
maintained after that point at 3 to 8 x 10^5 cells per ml
10 for two to three days and stained by indirect
fluorescence according to conventional methods. Cell
lines expressing high amounts of HLA class I will be
subcloned and selected cell populations will be sampled
15 for reinfusion into the patient.

Patients will be immunized weekly with 1 x 10^6 to
1 x 10^8 cells per dose intravenously; re-establishment of
tolerance to self will be monitored by sampling T-cells
from the recipient for proper T-cell development, i.e.
20 re-establishment of the high peak of CD45 or ICAM or
other memory cell markers, as well as for the lack of
cytotoxic T-cells to autologous antigen presenting cells
that have not yet been permanently transfected.

Transient Transfection

25 An alternative procedure for re-establishment of
tolerance to self will be to perform transient
transfections on freshly isolated antigen presenting
cells such as B cells. As previously outlined, freshly
isolated B cells will be purified from fresh heparized
30 blood; approximately 70 cc of blood will be drawn from
each patient. The B cells will be obtained from the non-
rosetting fraction of sheep red blood cell rosetted T-
cells, and these B cells will be enriched by panning out
the macrophages. Transient transfections will be
35 performed in the identical matter used for the permanent

- 23 -

transfections, except that the cells will be immediately
(within six hours) reinfused into the same patients at
higher doses, e.g., 1 x 10⁷ to 1 x 10⁸ cells per IV dose.
The cells will be able to be injected in an outpatient
5   clinic, since they represent cells from the same patient.
Treatment by Administration of Peptide Antigens

As mentioned earlier, where a defect in class I or
one of the genes encoding a protein involved in class I
presentation prevents proper presentation of a peptide
10   antigen on the surface of a cell, one strategy is to
provide that peptide. The first step in such therapy is
to identify the peptide or peptides which are not being
presented complexed to class I. This is done by
isolating cells from the patient, e.g., peripheral blood
15   lymphocytes, and eluting from the lymphocytes the
peptides complexed with HLA class I to form an elution
profile, according to standard techniques. Such methods
are described, e.g., in Madden et al. (1991), Nature,
353:321; Van Bleek et al. (1990), Nature, 348:213; and
20   Rudensky et al. (1991), Nature, 353:622.

The next step is to compare this elution profile
to a normal control, to identify the missing peptides in
the patient. Those peaks present in the control but
missing in the patient are then sequenced, and the
25   peptides synthesized by standard techniques and
administered as described above.

Tolerance to Non-Self Antigens

Tolerance to non-self antigens can be induced by
transfecting an HLA class I antigen presenting cell with
30   DNA encoding the protein to which tolerance is to be
induced and administering the transfected cells to a
patient. This would allow the introduced protein to be
artifically incorporated into the tolerance inducing
pathway of HLA class I presentation of self peptides.

- 24 -

The cell will present the foreign protein via class I as if it were endogenous, and the cells will induce tolerance to that antigen. This technique can be used to induce tolerance to allograft antigens prior to carrying

5   out the allograft, to inhibit rejection.

Other embodiments are within the following claims.

What is claimed is:

- 25 -

<u>Claims</u>

1. A cell presenting HLA class I, wherein said cell is transfected with DNA encoding a protein which is involved in the transport of endogenous proteins into the endoplasmic reticulum for HLA class I presentation.

2. The cell of claim 1, wherein said cell is an autologous cell.

3. The cell of claim 1, wherein said protein is an ATP-dependent transporter protein.

4. The cell of claim 3, wherein said DNA encodes RING 3, RING 4, HAM 1, Mtp 1, Y 3, HAM 2, or MAP 2.

5. A cell presenting HLA class I wherein said cell is transfected with DNA encoding a protein which is involved in the intracellular processing of endogenous proteins for HLA class I presentation.

6. Use of a substance which stimulates HLA class I expression in the preparation of a medicament for treating a mammal to inhibit an autoimmune disease.

7. The use of claim 6, wherein said substance is an interferon.

8. The use of claim 6, wherein said substance is a toxoid.

9. Use of a cell presenting HLA class I in the preparation of a medicament for treating a mammal to
5 inhibit an autoimmune disease.

10. The use of claim 9, wherein said mammal to be treated is a human patient and said cells presenting HLA class I are obtained from another human whose HLA class I expression level is higher than that of said patient.

10   11. Use of an antigen which forms a complex with an HLA class I molecule on the surface of a cell of a mammal to stabilize said class I and present said antigen to the immune system of said mammal in the preparation of a medicament for treating a mammal to inhibit an
15 autoimmune disease.

12. The use of claim 11, wherein said antigen is a peptide.

13. The use of claim 12, wherein said peptide is approximately 6 to 18 amino acids long, inclusive.

20   14. A method for testing a mammal for predisposition to develop an autoimmune disease, said

- 27 -

method comprising measuring HLA class I presentation on
cells of said mammal, a decreased level of presentation
indicating said predisposition.

15.   The method of claim 14, wherein said
5   autoimmune disease is type I diabetes.

16.   The method of claim 14, wherein said cells
are peripheral blood lymphocytes.

17.   A method for testing a mammal for
predisposition to develop an autoimmune disease,
10   comprising
obtaining a biological sample from said mammal,
and
determining, for said sample, whether there is a
defect in or deletion of a gene encoding a protein which
15   is involved in the transport of endogenous proteins into
the endoplasmic reticulum for HLA class I presentation.

18.   The method of claim 17, wherein said protein
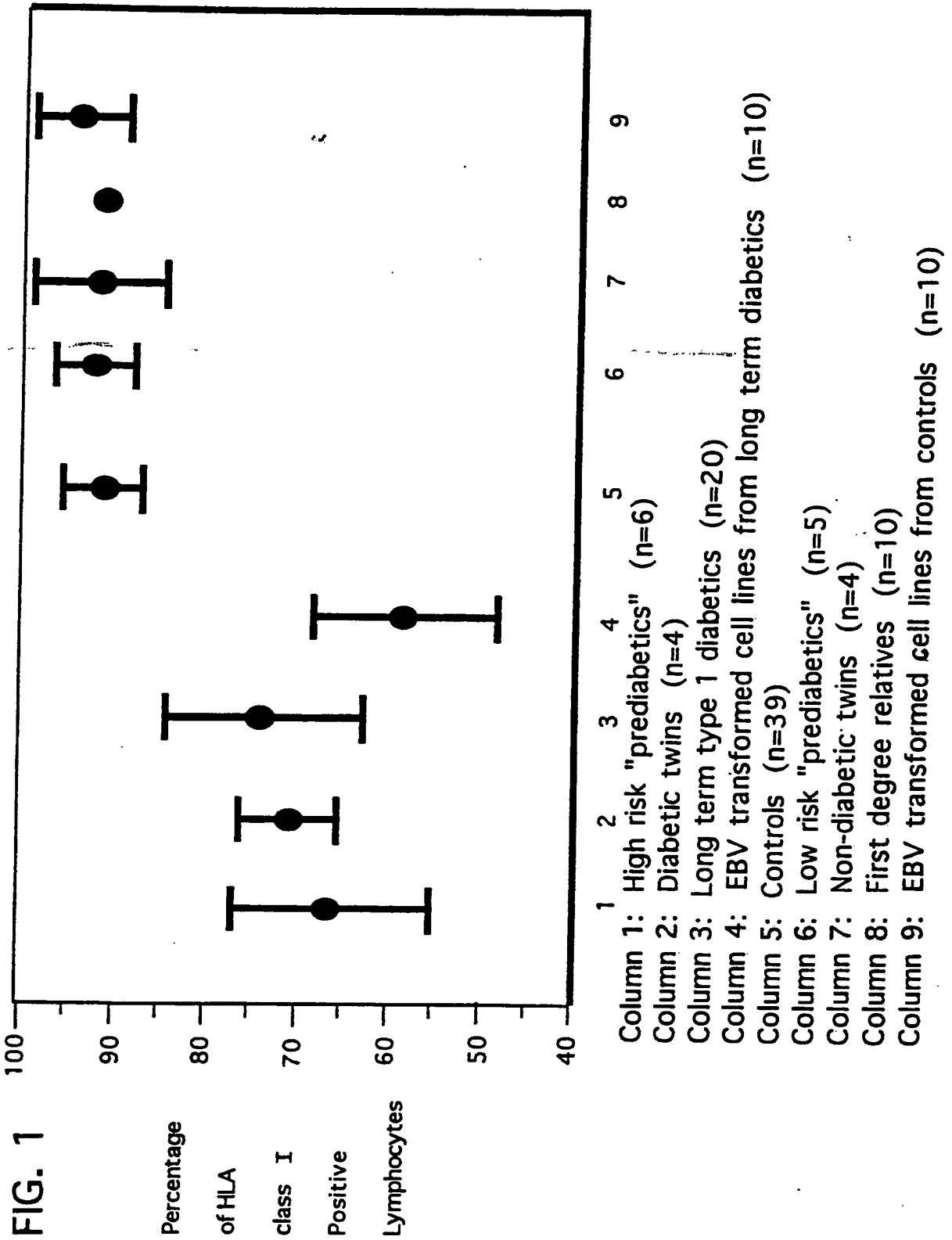is an ATP-dependent transporter protein.

19.   Use of a cell on which a protein is presented
20   bound to HLA class I in the preparation of a medicament
for treating a patient to induce tolerance to said
protein.

- 28 -

20. The use of claim 19, wherein said protein is a non-self protein present in an allograft.

21. The use of claim 19, wherein said cells are transfected with DNA encoding said protein.

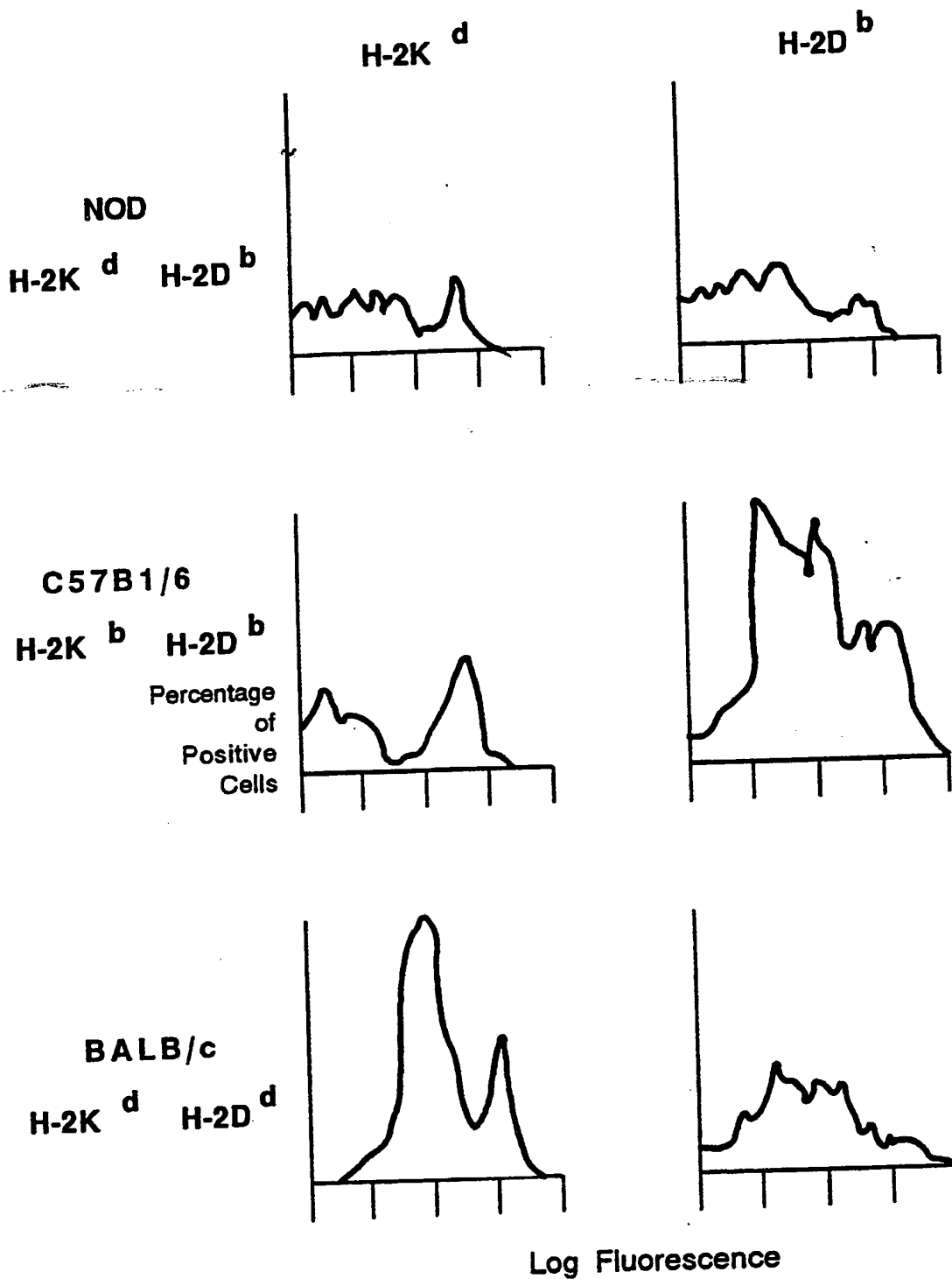5   22. The use of claim 21, wherein said cells are autologous B cells.
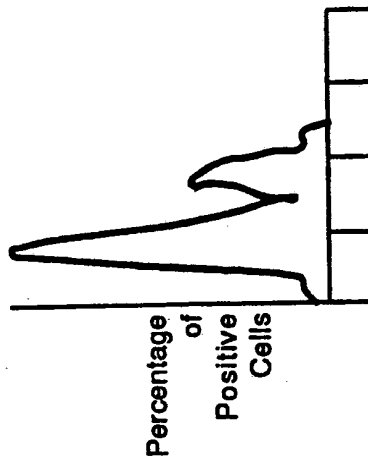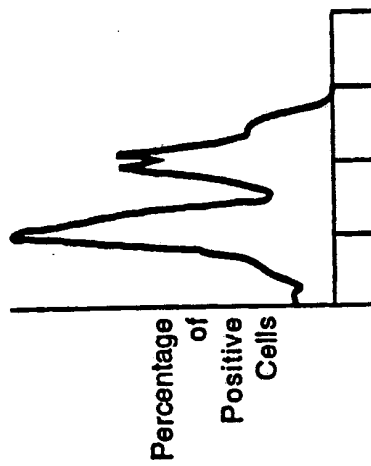
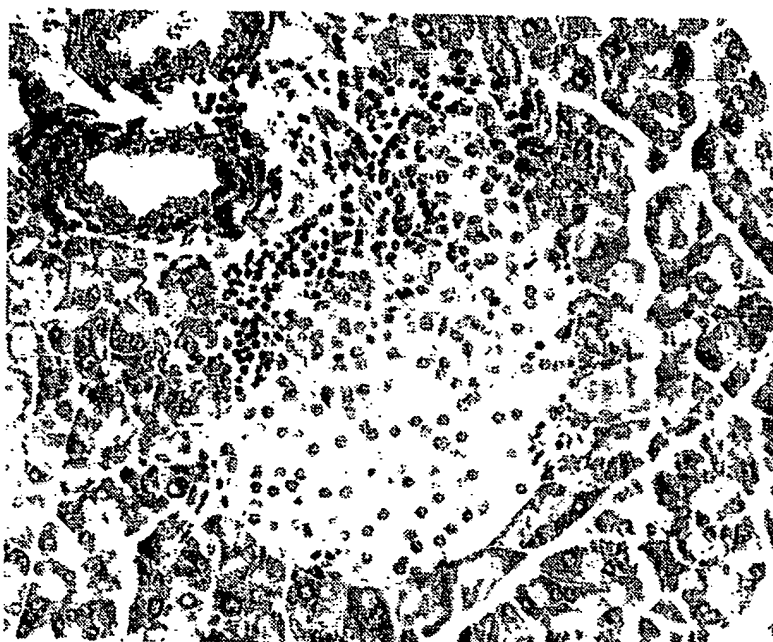FIG. 1

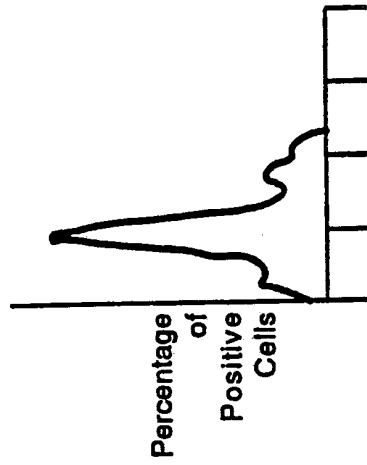Percentage of HLA class I Positive Lymphocytes

Column 1: High risk "prediabetics" (n=6)
Column 2: Diabetic twins (n=4)
Column 3: Long term type 1 diabetics (n=20)
Column 4: EBV transformed cell lines from long term diabetics (n=10)
Column 5: Controls (n=39)
Column 6: Low risk "prediabetics" (n=5)
Column 7: Non-diabetic twins (n=4)
Column 8: First degree relatives (n=10)
Column 9: EBV transformed cell lines from controls (n=10)

FIG. 2

FIG. 3

FIG. 4a

FIG. 4b

FIG. 4c

Percentage of Positive Cells

BALB/c

NOD

BETA 2 MICROGLOBULIN DEFICIENT

Log Fluorescence

FIG. 5

LANE 1 2 3 4 5 6 7 8 9

N B C N B C N B C N
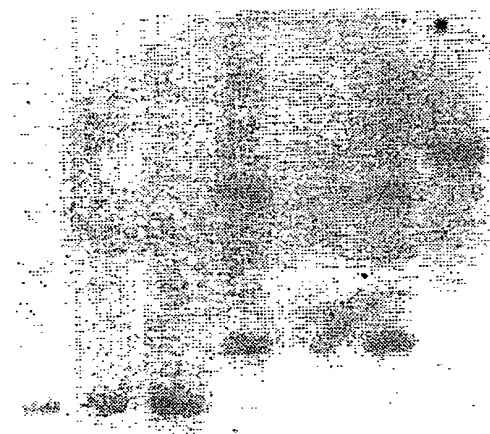1 5 5 5 16 16 16 19 19 19
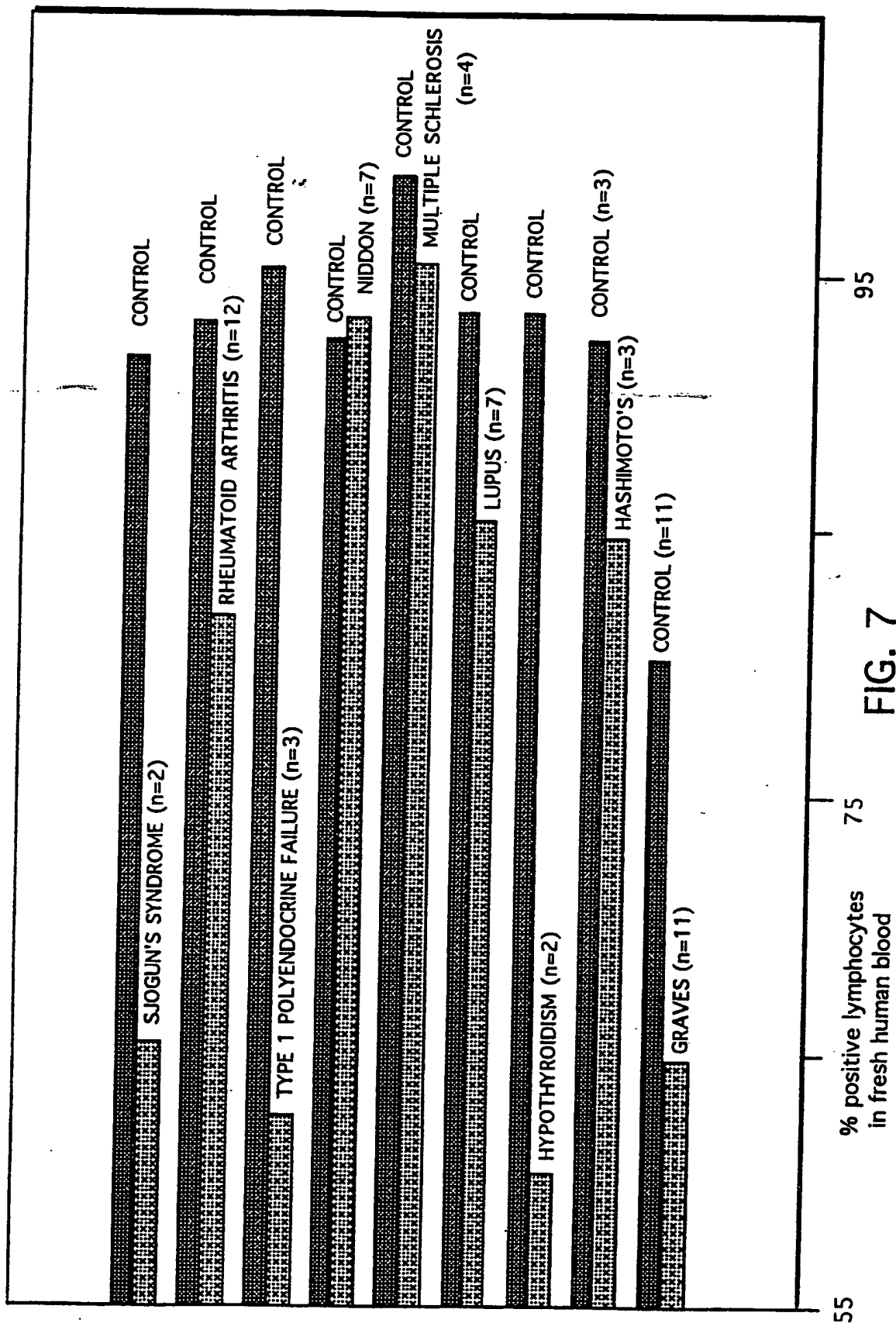3 4 5 6 7 8 9 10 11 12

FIG. 6

7/7

FIG. 7

INTERNATIONAL SEARCH REPORT

A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) :A61K 37/00
US CL :424/93

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/93

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

NONE

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, DIALOG CAS/HLA CLASS I PRESENTATION; AUTOIMMUNE DISEASE; ATP DEPENDENT TRANSPORTER PROTEIN; RING; IMMUNE DESTRUCTION OF SECRETORY CELLS; DENISE FAUSTMAN

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Clinical Immunology and Immunopathology, Volume 59, Issued 1991, Leiter et al, "Autoimmune diabetes in the Nonebese Diabetic Mouse: Supression of Immune Defects by Bone Marrow Transplantation and Implications for Therapy", pages 323-334, see entire article.	19 - 22
Y	Seminars in Medicine of the Beth Israel Hospital, Boston, Volume 317, Number 17, Issued 22 October 1987, Selden et al, "Regulation of Insulin-Gene Expression: Implications for Gene Therapy", pages 1067-1076, see entire article.	2, 9
Y	Chemical Abstracts, Volume 116, Issued 1992, Bikoff et al, "MHC Class I Surface Expression in Embryo-derived Cell Lines Inducible with Peptide or Interferon", see page 4853, column 1, abstract n. 116: 4858x, Nature 354(6350), 235-238, 1991.	6, 7, 11, 12, 13 - 15, 16, 17, 18
Y	Chemical Abstracts, Volume 115, Issued 1991, Spies et al, "Restored Expression of Major Histocompatibility Class I Molecules by Gene Transfer of a Putative Peptide Transporter", see page 594, column 2, abstract no. 115: 27312h, Nature, 351(6324, 323-324, 1991.	1, 3, 4, 5, 10

X	Further documents are listed in the continuation of Box C.		See patent family annex.

•	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be part of particular relevance		
"E"	earlier document published on or after the international filing date	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed	"&"	document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
15 OCTOBER 1992	OCT 1992

Name and mailing address of the ISA/	Authorized officer
Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231	SUZANNE ZISKA
Facsimile No. NOT APPLICABLE	Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Nature, Volume 348, Issued 20/27 December 1990, Trowsdale et al, "Sequences Encoded in the Class II Region of the MHC related to the 'ABC' Superfamily of Transporters", 741-744, see entire article.	08